BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

REMARKS

The present invention relates generally to providing fluorescent particles useful in determining analyte concentrations in aqueous solutions. The particles of the present invention incorporate dye pairs that are selected to provide advantageously large Stokes shifts (*i.e.*, the shift in wavelength between peak light absorption and peak fluorescent emission) through resonant energy transfer between the members of the dye pairs. *See, e.g.*, specification, page 13, lines 5-23. In addition, the fluorescent particles of the present invention can provide reduced quenching of the fluorescent signal. *See, e.g.*, specification, page 14, lines 12-18.

Claims 30-44 and 46 are presently pending in the application, with claims 30, 31, and 45 presently under consideration by the Examiner. Claim 30 is amended herein to incorporate the limitations of claim 45, and to explicitly acknowledge that the dye pairs are structurally distinct compounds; claim 45 is therefore cancelled herein. Notwithstanding the foregoing amendments, Applicants expressly reserve the right to prosecute subject matter no longer or not yet claimed in one or more applications that may claim priority to the instant application.

Applicants respectfully request reconsideration of the claims in view of the foregoing amendments and the following remarks.

Non Art-Related Remarks

35 U.S.C. § 112, Second Paragraph

Applicants respectfully traverse the rejection of claims 30, 31, 42, and 45, for allegedly being indefinite with regard to the term "particle."

When determining definiteness, the proper standard to be applied is "whether one skilled in the art would understand the bounds of the claim when read in the light of the specification." *Credle v. Bond*, 30 USPQ2d 1911, 1919 (Fed.Cir.1994). See also *Miles Laboratories, Inc. v. Shandon, Inc.*, 27 USPQ2d 1123, 1127 (Fed.Cir.1993) ("If the claims read in the light of the specification reasonably apprise those skilled in the art of the scope of the invention, § 112 demands no more.").

The rejection asserts that because "Applicants do not specify the size, shape, composition or any other distinguishing physical or chemical properties of a 'particle'... it is not clear how this term further limits the claimed invention." Office Action, pages 12-13. Applicants respectfully submit that this analysis fails to consider the claim term in view of the understanding of the skilled artisan and in light of the instant specification.

The term "particle" is commonly used by those of skill in the immunoassay art to refer to a solid phase that is dispersible in an assay medium, as opposed to a solid phase such as a membrane, coated tube, microtiter dish, *etc.*, which is not dispersible. For example, Chapter 4 of *Principles and Practice of Immunoassay*, Price and Newman, eds., Stockton Press, New York, 1991 (attached herewith for the convenience of the Examiner) describes the skilled artisan's understanding of the term "particle" in precisely this context (see especially pages 86-90). Applicants note that this chapter demonstrates that the artisan understands the scope of the generic term "particle" without need to specify "the size, shape, composition or any other distinguishing physical or chemical properties." In addition, numerous particles for use as solid phases were readily commercially available. For example, the instant specification describes the use of commercially available "carboxyl-modified latex particles" for preparation of the claimed particles.

Although Applicants maintain that the term "particle" is not indefinite, in an effort to advance prosecution, Applicants have amended claim 30 herein to specify a size range for the particles of the instant claims. The remaining characteristics (shape, composition, *etc.*) of the claimed particles (other than the components specified in the claims) need not be specified in the claim for the claim to meet the requirements of Section 112, Second Paragraph.

In view of the common understanding and use of the term "particle" in the art, Applicants respectfully submit that the rejection be reconsidered and withdrawn.

Art-Related Remarks

35 U.S.C. § 102

Applicants respectfully traverse the rejection of claims 1 and 45 as allegedly being anticipated by Wheeler *et al., J. Am. Chem. Soc.* 106: 7404-10 (1984). As an initial matter, Applicants request clarification of the rejection, as claim 1 is not pending in the application. Also, claim 45 has been cancelled and its limitation incorporated into claim 30. Nevertheless, the following response is a good faith attempt to address the basis of the rejection for any of the pending claims.

In order to anticipate a claim, a single prior art reference must provide each and every element set forth in the claim. *In re Bond*, 15 USPQ2d 1566, 1567 (Fed. Cir. 1990). *See also*, MPEP §2131. The Examiner bears the initial burden of establishing a *prima facie* case of anticipation. Only once that *prima facie* case has been established does the burden shift to the applicant to rebut the *prima facie* case. *See, e.g., In re Morris*, 127 F.3d 1048, 1054 (Fed. Cir. 1997).

The anticipation rejection is based upon a fundamentally flawed interpretation of the claims; specifically, the rejection asserts that the "first compound" and "second compound" of the instant claims may be interpreted to be two molecules of the same compound. Office Action, page 13. Applicants respectfully submit that this interpretation of the claims is unreasonable, as construing the claim language in this manner "would render meaningless [an] express claim limitation[ ]." *Unique Concepts, Inc. v. Brown*, 939 F.2d 1558, 1563 (Fed. Cir. 1991); *see also, Texas Instruments Inc. v. United States Int'l Trade Comm'n*, 988 F.2d 1165, 1171 (Fed.Cir.1993) (rejecting a patentee's proffered claim construction because it "would render the disputed claim language mere surplusage."). Specifically, the recitation of a distinct "first compound" and "second compound" in the claims is rendered meaningless if each merely refers to the same compound.

Nevertheless, in an effort to advance prosecution, Applicants have amended the claims to indicate that the first compound differs in structure from said second compound. This limitation does not further limit the claim, but merely represents a further acknowledgement of the distinct

nature of the "first compound" and "second compound" already recited in the claims. Thus, it is respectfully submitted that the rejection for anticipation fails on this basis alone and should be withdrawn.

Furthermore, the anticipation rejection is also based on an unsupported assertion that "small particles are generally used in chromatography." Office Action, page 14. The Wheeler reference refers to chromatography on alumina, but does not even indicate if the alumina is in the form of a particle. A rejection must be based on objective evidence of record, and not upon bare opinion of the Examiner. Applicants respectfully request that the Examiner cite some evidence for this assertion, particularly in light of the foregoing claim amendments that recite a specific size range for the instantly claimed fluorescent particles.

In view of the foregoing amendments and remarks, Applicants respectfully submit that the rejection under 35 U.S.C. § 102 is moot or without basis, and request that it be reconsidered and withdrawn.

35 U.S.C. § 103

Applicants respectfully traverse the rejection of claim 30, 31, and 45 as allegedly being unpatentable under 35 U.S.C. § 103(a) over the combination of Sounik *et al.*, EP 0391284; Wheeler *et al., J. Am. Chem. Soc.* 106: 7404-10 (1984); Rembaum et al., U.S. Patent 4,326,008; Schwartz et al., U.S. Patent 4,609,689; Oguri et al., U.S. Patent 5,254,887; Wang et al., U.S. Patent 4,420,568; Fino et al., U.S. Patent 4,476,229; and an allegation by the Examiner that the specification provides an admission of the contents of the prior art. Applicants submit that no *prima facie* case of obviousness has been established.

To establish a *prima facie* case of obviousness, three criteria must be met; there must be some motivation or suggestion, either in the cited publications or in knowledge available to one skilled in the art, to modify or combine the cited publications; there must be a reasonable expectation of success in combining the publications to achieve the claimed invention; and the publications must teach or suggest all of the claim limitations. *In re Vaeck*, 20 USPQ2d 1438 (Fed. Cir. 1991); MPEP § 2142. In analyzing obviousness, the Court of Appeals for the Federal Circuit has repeatedly cautioned that:

> [t]he factual inquiry... must be based upon objective evidence of record.... [T]he best defense against the subtle but powerful attraction of a hindsight-based obviousness analysis is rigorous application of the requirement for a showing of the teaching or motivation to combine prior art references.... [P]articular findings must be made as to the reason the skilled artisan, with no knowledge of the claimed invention, would have selected these components for combination in the manner claimed.

In re Sang-Su Lee, 277 F.3d 1338, 1343 (internal citations omitted).

Furthermore, in order to establish a *prima facie* case of obviousness, the examiner must establish that some objective teaching, suggestion or motivation in the applied prior art taken as a whole, and/or knowledge generally available to one of ordinary skill in this art, would have led that person to the claimed invention as a whole, including each and every limitation of the claims, without recourse to the teachings in the applicant's disclosure. *See generally, In re Rouffet*, 149 F.3d 1350, 1358 (Fed. Cir. 1998); *Pro-Mold and Tool Co. v. Great Lakes Plastics Inc.*, 75 F.3d 1568, 1573 (Fed. Cir. 1996); *In re Fine*, 837 F.2d 1071, 1074-76 (Fed. Cir. 1988); *In re Dow Chem. Co.*, 837 F.2d 469, 473 (Fed. Cir. 1988).

In contrast to this established principle, the present rejection is based squarely upon hindsight using the teachings in the instant specification. Specifically, the Examiner contends that the Sounik *et al.* publication discloses certain phthalocyanine, naphthalocyanine, and anthranylocyanine dye mixtures. But with regard to the incorporation of such dye mixtures into particles, the rejection relies solely on the instant specification. Office Action, page 8 ("Here, Applicants claimed 'particles' are disclosed by the admission of prior art in the specification"). According to the Examiner, "Applicants' specification admits that '[o]ne skilled in the art will recognize that water insoluble dyes can be incorporated into latex particles as described in U.S. Pat. Nos. 4,326,008, 4,609,689 and 5,254,887.'" Office Action, page 3.

The statement in the specification on which the rejection relies, however, reflects that which the skilled artisan will understand based on Applicants' own teachings described in the instant specification. It does not constitute, as the Examiner apparently believes, any admission concerning what the skilled artisan might understand based solely on the prior art. The fact that the present Applicants recognized that certain methods disclosed in the prior art could be used to

incorporate the specific dye pairs of the instant claims into particles cannot be used to establish a *prima facie* case of obviousness.

The rejection continues in a similar vein, asserting that "Applicants further admit that small particle sizes between 0.1 nm and 5000 nm were known in the art at the time of filing" referring to Applicants' discussion of the use of certain particle sizes useful in fluorescence polarization assays. Office Action, paragraph bridging pages 3 and 4. But again, the fact that Applicants recognized that the particles described in the instant claims may find use in certain types of assays in accordance with the claimed invention cannot be used to establish a *prima facie* case of obviousness for the instant claims.

Because the present obviousness rejection rests entirely upon Applicants' own disclosure rather than any teaching of the prior art, the rejection fails to meet the well established requirements for establishing a *prima facie* case of obviousness.

In addition to this misplaced reliance upon Applicants' own disclosure for certain claim elements, the rejection contains numerous other flaws that separately preclude the establishment of a *prima facie* case of obviousness. To begin an analysis of these flaws, the following table provides a compilation of the claimed subject matter that is missing from the primary Sounik *et al.* publication:

Claim language (presently pending):	Deficiencies:
30. A fluorescent particle comprising:	The cited publication does not disclose any particles. Rather, the publication refers to, *e.g.*, mixing dyes with polymers and heating until a melt phase is formed, which can be molded into "suitable shapes." Furthermore, the publication is silent on the fluorescence of compositions, and nothing of record indicates that dyes, when formed by melting into polymers in this fashion, would retain any fluorescent properties.
a. a first compound selected from the group consisting of silicon phthalocyanine bis(dimethylhexylvinylsilyloxide) and silicon phthalocyanine bis(trihexylsilyloxide); and	The cited publication does not disclose any compounds having the claimed structures, much less two such compounds in the same particle, as required by parts (a) and (b) of the claim.
b. a second compound that is a bis(dimethylhexylvinylsilyloxide)-substituted or bis(trihexylsilyloxide)-substituted phthalocyanine, naphthalocyanine, or anthranylocyanine derivative, or a bis(dimethylhexylvinylsilyloxide)-substituted or bis(trihexylsilyloxide)-substituted hybrid phthalocyanine derivative, wherein the size of said fluorescent particle is between 0.1 nm and 5000 nm, and wherein said first compound differs in structure from said second compound.	

Acknowledging that the Sounik *et al.* publication fails to disclose the specific compounds recited in the instant claims, the rejection relies on the secondary Wheeler *et al.* publication, which allegedly discloses silicon phthalocyanine bis(trihexylsiloxide) and silicon naphthalocyanine bis(trihexylsiloxide) as individual molecules in solution (and not as components of a particle) in order to provide each of the individual components of the instantly claimed invention.

The rejection then offers an assortment of possible motivations for combining the specific compounds allegedly disclosed in the secondary Wheeler *et al.* publication with the discussion of "dye mixtures" allegedly disclosed by the Sounik *et al.* publication, in order to provide the particles of the instant claims.

The first two of these alleged motivations are again admittedly taken straight from Applicants' own disclosure: "because Applicants' specification admits that '[o]ne skilled in the art will recognize that water insoluble dyes can be incorporated into latex particles'"; and "because 'water insoluble dyes can be made useful by incorporation into latex particles for visualization in a variety of assay formats.'" Office Action, page 5. This reliance on Applicants' own specification to establish obviousness, which pervades the rejection, again runs afoul of the admonishment that the various claim elements, as well as a motivation to combine publications to arrive at the claimed invention, must be found without recourse to the teachings in the applicant's disclosure.

The third possible motivation offered in the rejection is that "according to Wheeler et al '[t]he presence of the trialkylsiloxy groups on the central Si atom leads to relatively high solubility in these compounds and permits studies of solutions of them at the millimolar level' ... which would be beneficial in assays." Office Action, page 5. What is left unstated, however, is why solubility and a benefit in assays would lead one skilled in the art to combine the specific compounds allegedly disclosed in the secondary Wheeler *et al.* publication with the "thin film optical media[,]... optical light switch and light modulator devices... comprising a thin film of a tetrazaporphin composition" for which the dye mixtures allegedly disclosed by the Sounik *et al.* publication are used. No possible relevance for solubility and a benefit in assays in that context has been offered. Moreover, no reasoning has been offered for why the skilled artisan would consider solubility to be of any advantage when considering to incorporate dye mixtures into fluorescent particles.

These fundamental flaws in the rejection are further emphasized by the Examiner's reply in the Office Action to Applicants' previous responses on the merits of the rejection. For example, the Examiner first dismisses Applicants' remarks by asserting that "one cannot show nonobviousness by attacking references individually." Office Action, page 8. Applicants

respectfully submit that this assertion, while perhaps true, is beside the point. The foregoing remarks do not attack references individually but rather serve to demonstrate the flaws in basis for combining publications offered in the Office Action.

With regard to the fact that neither the Sounik *et al.* publication nor the secondary Wheeler *et al.* publication disclose any particles, fluorescent or otherwise, the Examiner takes the position that the newly added secondary publications (Rembaum et al., U.S. Patent 4,326,008; Schwartz et al., U.S. Patent 4,609,689; Oguri et al., U.S. Patent 5,254,887; Wang et al., U.S. Patent 4,420,568; and Fino et al., U.S. Patent 4,476,229), which are discussed in the instant specification, are "admitted" by the specification to disclose particles. Office Action, page 8. The irrelevance of this argument, and the inapplicability of using Applicants own disclosure to the establishment of a *prima facie* case of obviousness was discussed in detail above.

The rejection also takes the position that "it is not clear what is meant by a 'particle" and, as a result, the mixtures of compounds disclosed by Wheeler et al. and Sounik et al also constitute 'particles.'" The failure of the Examiner to properly interpret the term "particle" in accordance with its meaning in the art is discussed in detail above with regard to the rejection under 35 U.S.C. § 112. Moreover, nowhere does the rejection provide any indication as to how the term "particle" is being interpreted by the Examiner. For this reason alone, no *prima face* case of obviousness has been established. *See*, MPEP § 2143.03 (it is improper to rely on speculative assumptions regarding the meaning of a claim and then base a rejection under 35 U.S.C. 103 on those assumptions).

The Examiner responds to Applicants' arguments that no fluorescent particles are disclosed (in the prior art references) with the assertion that "[f]luorescent particles are disclosed" based on this unstated (and hence unrebuttable) interpretation of the term "particle" that sweeps in whatever is disclosed by the Wheeler et al. and Sounik et al. publications. Office Action, page 9. The Examiner also responds by stating that "the preamble of claim [sic] (i.e., where the term 'fluorescent' is used) is usually not afforded patentable weight." *Id.* This, however, is inconsistent with the Examiner's consideration of the term "particle" as a limitation, despite the fact that it too appears only in the preamble of the claims. Why the modifier "fluorescent" that appears immediately before the term "particle" in the claims should not also be

considered a part of the claim is unclear. Preamble language will be considered an element of the claim if it recites not merely context, but rather the essence of the invention. *See, e.g., Boehringer Ingelheim Vetmedica, Inc. v. Schering-Plough Corporation*, 320 F.3d 1339, 1345 (Fed. Cir. 2003). In the present case, the essence of the invention is the provision of the claimed fluorescent particles. Furthermore, the claim as now amended refers to "said fluorescent particle" in the body of the claim. A preamble phrase is limiting when it provides antecedent basis for terms or limitations in the claim body. *See Catalina Marketing International, Inc., v Coolsavings.com, Inc.*, 289 F.3d 801, 810-811 (Fed. Cir. 2002) (preamble phrase not limiting in claim where only mentioned in preamble but limiting in claim where phrase included in the claim body); *In re Higbee* 527 F.2d 1405, 1407 (CCPA 1976) ("casing" in preamble limiting because "said casing" follows the preamble.").

Applicants respectfully submit that when the term "particle" is properly interpreted, it is apparent that neither the Sounik *et al.* publication nor the secondary Wheeler *et al.* publication disclose any particles, fluorescent or otherwise, particularly within the size range recited in the claims as amended herein, and that no motivation has been established to provide any fluorescent particles recited in the instant claims.

Applicants also noted in their prior remarks that, in order to arrive at the instant claims, the rejection cannot simply place <u>one</u> of the individual molecules allegedly disclosed by the Wheeler *et al.* publication into a particle; instead, the Examiner must provide a motivation to place <u>two</u> of the molecules disclosed by the Wheeler *et al.* publication in a single fluorescent particle in order to provide the instantly claimed invention, despite the fact that the each cited publication is silent as to providing any such combination. Thus, the artisan must:

(i) select a molecule in the Sounik *et al.* publication comprising a ligand (Z),

(ii) modify the ligand to provide a trihexylsiloxide group according to the disclosure of the Wheeler *et al.* publication,

(iii) select two such ligands (Z) on the molecule,

(iv) combine the molecule with a second molecule that is also selected to include each of (i)-(iii), and

(v) provide this combination as a fluorescent particle.

In response, the Examiner asserts that "'motivation' does not have to be provided for 'each' molecule as Applicants contend because 'there is no requirement that the prior art provide the same reason as applicant to make the claimed invention.'" Office Action, page 8. However, this begs the point of Applicants' arguments. The instant claims refer to a particle having two compounds, each of which comprises dimethylhexylvinylsilyloxide or trihexylsilyloxide. The only two such molecules in the asserted *prima facie* case are the trihexylsilyloxide-containing molecules allegedly disclosed in the Wheeler *et al.* publication. Whatever motivation is proposed by the Examiner, it must include a reason that the skilled artisan, with no knowledge of the claimed invention, would have selected the claimed components for combination in the manner claimed; that is, there must be a motivation to provide two different molecules, each of which comprises dimethylhexylvinylsilyloxide or trihexylsilyloxide, in a single fluorescent particle. By failing to provide a motivation to combine two of the molecules allegedly disclosed by the Wheeler *et al.* publication into a single particle, the Examiner has failed to establish a *prima facie* case of obviousness.

The Examiner also asserts that two such molecules need not be provided in a single particle because the first and second compounds recited in the claims "can be the same." Office Action, page 10. The flaws in this assertion were discussed in detail above with regard to the rejection under 35 U.S.C. § 102. This argument is untenable in view of the express claim language for compounds that "differ" structurally.

Applicants respectfully submit that, when the cited publications are properly considered, it is apparent that any motivation to modify or combine the cited publications in order to provide the instant claims can only be gleaned in hindsight using the instant specification as a guide. In the absence of the teachings of the instant application, the skilled artisan would not have a motivation to combine the publications as the Examiner contends. Because a motivation to modify the cited art must be found in the prior art, and not in applicant's own disclosure, no *prima facie* case of obviousness has been established. *See, e.g., In re Vaeck*, 20 USPQ2d 1438 (Fed. Cir. 1991); MPEP § 2142.

Further compounding the flaws in the rejection, the Examiner also exhibits a misunderstanding of how evidence of unexpected properties is to be considered. Applicants have submitted evidence that the claimed fluorescent particles exhibit unexpected properties that overcome any *prima facie* case of obviousness that may have been established by the Examiner. Specifically, when the dye pairs recited in the claims are included in a single fluorescent particle, fluorescence energy transfer ("FET") occurs between the members of the dye pair. In FET, all or a portion of the energy absorbed by one member of a dye pair (the "donor") is not emitted as detectable emission light by that member; instead, the energy is "transferred" to the second member of the dye pair (the "acceptor") and emitted at the longer emission wavelength of the acceptor. As described in the instant specification on page 40, lines 10-19, FET was confirmed in the particles of the instant invention by liberation of the dyes and measuring the loss in emission intensity of the acceptor dye. The FET parameters of the particles of the present invention are described in detail in the Example beginning on page 43, line 21. In addition, as described in the instant specification on page 87, incorporation of tetraazaporphyrin dyes in particles can result in dramatic quenching of the fluorescence signal. The use of axial ligands such as bis(dimethylhexylvinylsiloxide) can dramatically reduce this quenching.

The Examiner's response that "Applicants claims are not limited to dye pairs that undergo fluorescence energy transfer" (Office Action, page 10) is incorrect. The property of FET is inherent in the claimed combination of fluorescent compounds. Thus, the features upon which Applicants rely most certainly are recited in the instant claims. These advantages are described in detail in the instant specification, and must be considered by the Examiner. *See*, MPEP § 2144.08(II)(B) (it is error not to consider evidence presented in the specification). Applicants respectfully submit that, by failing to properly consider Applicants' evidence in this regard, the Examiner has not considered the record as a whole. Because no *prima facie* case of obviousness has been established by the Examiner, or, in the alternative, because any *prima facie* case of obviousness has been rebutted by a showing of unanticipated properties of the claimed invention, Applicants respectfully request that the Examiner reconsider and withdraw the rejection under 35 U.S.C. § 103.

Obviousness-type double patenting

Applicants continue to acknowledge the obviousness-type double patenting rejection of claims 30-31, 42, and 45. Should the claims be found allowable as presently written, a terminal disclaimer will be submitted.

CONCLUSION

In view of the foregoing remarks, Applicants respectfully submit that the pending claims are in condition for allowance. An early notice to that effect is earnestly solicited. Should any matters remain outstanding, the Examiner is encouraged to contact the undersigned at the address and telephone number listed below so that they may be resolved without the need for additional action and response thereto.

Respectfully submitted,

Date June 10, 2004

By 

FOLEY & LARDNER LLP
Customer Number: 30542
Telephone: (858) 847-6722
Facsimile: (858) 792-6773

Barry S. Wilson
Registration No. 39,431
For
Richard J. Warburg
Registration No. 32,327

Principles and Practice of Immunoassay

Editors
Christopher P. Price
David J. Newman

M
stockton
press

Published in the United States and Canada by
STOCKTON PRESS, 1991
15 East 26th Street,
New York, N.Y. 10010, USA

ISBN 1-56159-020-7

First published in the United Kingdom by
MACMILLAN PUBLISHERS LTD, 1991
Distributed by Globe Book Services Ltd
Brunel Road, Houndmills
Basingstoke, Hants RG21 2XS

Reprinted in 1992

British Library Cataloguing in Publication Data

Price, Christopher P.
Principles and practice of immunoassay.
I. Title II. Newman, David J.
543.0028

ISBN 0-333-51406-8

Printed in Great Britain

4 Separation techniques

INTRODUCTION

All heterogeneous immunoassays require a procedure to separate the bound labelled ligand from the free. There have been several reviews focussing on this important aspect of immunoassay, namely Ratcliffe in 1974 (1), a multi-author section in the book edited by Hunter and Corrie in 1983 (2) and more recently by Gosling in 1990 (3). Here we shall summarise the salient features that are expected of a separation technique, and with passing reference to historical approaches, focus on those in current use with concluding comments on some future possibilities.

REQUIREMENTS OF A SEPARATION TECHNIQUE

An ideal separation technique will ensure complete separation of free and bound labelled antibody or antigen, **without** modulation of the primary antigen:antibody reaction and whilst remaining indifferent to the composition of the reaction matrix. If separation is incomplete misclassification errors arise which result in assay bias and, if there is a lack of robustness, there will be an increase in assay imprecision. Such a lack of 'efficiency' in a separation technique can be due to physical trapping of the free ligand, adsorption of the free ligand to the assay tube, impurities in the labelled ligand or simply to a failure to remove all the supernatant liquid. The conventional measure of this 'efficiency' is the assay non-specific-binding (NSB).

If the separation technique interferes with the primary antigen:antibody reaction, and this can be by changing the rate of attainment of equilibrium or the equilibrium position reached, then a bias will be introduced, that may also be variable (*e.g.* dependent on reaction time) and thereby increase imprecision. As immunoassays are performed in a variety of biological matrices *e.g.* whole blood, serum, plasma, urine, faeces, CSF, *etc.* a separation technique should be robust to the influence of 'matrix' effects.

In addition to the above fundamental attributes any separation system must

also be practical (*i.e.* simple, quick and cheap), preferably be readily automated and be applicable to a wide range of analytes.

HETEROGENEOUS IMMUNOASSAY

Heterogeneous vs homogeneous

The lack of a separation step is the mark of a homogeneous assay and this can be advantageous in terms of the speed and convenience of an assay. However the inclusion of such a separation step provides the opportunity for introducing a wash step which has many advantages for improving assay detection limits particularly in those situations where the NSB is critical to overall assay performance. Thus although homogeneous assays have proved extremely useful for analytes, in the micromolar concentration range and above, it is to heterogenous systems that we must turn to achieve the sensitivity to measure analytes such as thyrotropin (TSH) in the pico-femtomolar concentration range.

Limited reagent vs excess reagent

As is discussed by Ekins in Chapter 5, and by other authors in this volume, it is the excess reagent (or non-competitive) assay systems that are the most influenced in their performance by NSB. By contrast the performance of limited reagent (or competitive) assays is determined more by the equilibrium constant of the reagent antibody. Thus the precision and detection limit of excess reagent assays is significantly improved by the ability to wash the immunoaggregate; the development of these systems has thus been associated with the developemnt of solid-phase separation technologies which began in the early 1960's (4,5).

CLASSIFICATION OF SEPARATION SYSTEMS

Separation systems can be classified as shown in Table 4.1. and represents a chronicle of assay development and increasing sophistication. The first separation procedures in immunoassay were performed using the physicochemical differences between the immunoglobulin and the antigen. This approach, if size related, was limited to assays for smaller molecules *e.g.* haptens. Chromatography (TLC initially) and electrophoresis gave high NSB's, in the region of 20% or more, of the total activity, and were

time consuming and slow. These techniques were superseded by the dextran-coated charcoal and fractional precipitation approaches which were widely used in the 1970's and even the early 1980's. Although they were more practicable they still gave unacceptably high NSB's, in the range of 10-20%, required the use of a refrigerated centrifuge and, in the case of dextran-charcoal, could significantly interfere with the primary antigen:antibody reaction. Although the fractional precipitation techniques enhanced the molecular size range for analytes that could be measured, these techniques all suffered from an essential lack of specificity for the antibody. Their major advantage, and the reason that they survived so long, was that they used cheap reagents.

Table. 4.1. Classification of separation techniques.

PRINCIPLE	EXAMPLES
Physicochemical characteristics of antibody or antigen.	electrophoresis:— charge chromatography:— molecular size
Adsorption	coated charcoal:— binds free fraction ion-exchange resins:— charge hydroxyapatite:— charge
Fractional precipitation	ethanol (usually at -20°C) polyethylene glycol, PEG, (at around 200 g/L) cross-linked dextran (at around 200 g/L) ammonium or sodium sulphate (at around 200 g/L or greater)
Immunological i) liquid-phase ii) solid-phase	anti-species secondary antiserum (± PEG) primary antibody:— particles, membranes, tubes, microtitre plates, paddles *etc.* secondary antibody:— particles, membranes, tubes, microtitre plates, paddles *etc.*

The introduction of secondary anti-species antisera was a major advance in finally introducing specificity for the essential component of the

immunoassay reaction (6), bringing NSB's down to the 5-10% region. The second antibody acted to increase the size of the immune-complex to enable easier separation by direct centrifugation. The essential immunological specificity of this interaction also ensured that there was no interference with the primary antigen:antibody reaction. The downside was that they were relatively expensive and time consuming, requiring overnight reactions and refrigerated centrifuges. This resulted in the development of a more economical approach to the use of secondary antibodies by combining them with a low concentration of PEG (40 g/L), thus reducing antisera usage, reaction time and the need for a refrigerated centrifuge and further reducing the NSB to the region of 2% (7). This review will focus on the continuing development of immunologically based separation systems.

IMMUNOLOGICAL SEPARATION SYSTEMS

Anti-species second antibodies became available in the late 1970's and early '80's, and they are now widely available as both poly- and monoclonal antibodies. The ability to couple secondary antibodies to a solid-phase, and more recently the primary antibody, has led to immunological separation systems being almost exclusively used in modern heterogeneous immunoassays. In an extension to the immunological specificities of primary and secondary antibodies there is now the potential to use the bacterial proteins Protein A or Protein G, both having specific binding sites for the F_c region of immunoglobulins; lectins and the streptavidin:biotin reaction are other examples of separation systems of excellent specificity (8,9).

The use of liquid-phase second antibody systems, as described above, has the disadvantage of requiring relatively large amounts of antibody, long reaction times and the use of a refrigerated centrifuge. Using a liquid phase system also has the disadvantage that washing of the precipitate requires great care. The development of particulate and subsequently tube and microtitre plate solid-phases has enabled frequent washing ultimately without the requirement for a centrifuge and reducing NSB's to a fraction of 1%.

Solid-phase immunological separation systems

An enormous range of solid-phase supports is now available, ranging from

microparticulate to microtitre plates and membranes (Table 4.2). The relatively slow introduction of these techniques (they were known in the early 1960's (10) but not in wide spread use until the 1980's) was due to difficulties in producing reliable coating/coupling procedures to link the antibodies to the solid-phase. This was particularly a problem with tube and plate supports and as a result particulate solid-phases were the first to receive widespread acceptance as their large surface area enabled more reproducible reaction conditions (11-14).

Table 4.2. Solid-phase supports for use as separation systems in immunoassay.

SIZE	EXAMPLE	ADVANTAGE	DISADVANTAGE
Small particle (<20µm)	Latex. Microcrystalline-cellulose. Fine porus glass. Some magnetic particles.	Dispensing as for liquids. Agitation not required. High antibody binding capacity.	Centrifugation required (unless used with a membrane capture). Long magnetic precipitation.
Medium particle (< 1mm)	Sepharose beads. Sephacryl beads. Sephadex beads.	Centrifugation not required. Short magnetic separation. Moderate antibody binding capacity.	Agitation required. Slower reaction kinetics than above.
Single particle (> 1mm)	Polystyrene. Nylon .	Centrifugation not required. Agitation not required.	Some variability in antibody coupling. Lower antibody binding capacity. Difficulty in dispensing. Poor reaction kinetics.
Solid surfaces	Coated tubes. Dipsticks. Microtitre plates. Membranes.	Centirifugation not required. Agitation rare. No dispensing of reagent. Simplest to use.	Variability in antibody coupling. Lowest antibody binding capacity. Slowest reaction kinetics.

Coupling of antibody/antigen to a solid-phase: It should be remembered from the outset that it is possible to couple both the antibody

and the antigen to a solid-phase and both approaches have been used in heterogeneous immunoassays. A wide range of coupling technologies have been used ranging from simple non-covalent adsorption, to a plethora of covalent coupling methodologies (11-15). They range from the use of toxic agents such as cyanogen bromide (13), which require the appropriate facilities and experience to use, to less toxic agents which enable smaller laboratories to prepare their own reagents with ease (14-16). Whole antiserum, IgG preparations, affinity purified antibodies, ascites fluid and antibody fragments have all been succesfully coated onto solid-phase supports. Monoclonal antibodies tend to be more variable in the reaction conditions required, than polyclonal antisera, but their ready availability in large quantities make their use much more attractive.

One aspect of coupling immunoreagents to solid phases is that their stability is enhanced. Thus latex particle coupled antibody is stable for over a year stored as a liquid reagent (17) and if solid phases are dried and stored dessicated the antibody is stable almost indefinitely (18). Non-covalently bound antibody is less resistant to desorption than covalently bound but care is required in the selection of a benign coupling technique that does not damage the antibody in its own right (19). Further discussion on the coupling of proteins to solid phase surfaces can be found in Chapter 16.

Primary vs secondary antibody vs antigen

The advantages of coupling a secondary antibody to a solid-phase are: no effect on the kinetics of the primary antigen:antibody reaction, applicable to a wide range of assays, efficient use of primary antibody. If the primary antibody is coupled to a solid-phase this restricts the movement of the antibody and can considerably slow the rate of reaction with the antigen, also the solid-phase is only suited for one assay (20). The coupling process, whether covalent or non-covalent, will result in partial denaturation of the antibody molecule and it has been shown that the affinity constant of an antibody may change on coupling to a solid-phase (21). This loss of affinity/avidity can thus result in an increased usage of precious primary antibodies, and this limited the usage of primary antibody solid-phases until the advent of monoclonal antibodies rendered this a financial rather than a volume consideration. The continuing dilemma with solid phase technologies is choosing between high antibody binding capacity and low NSB, as in general there is an inverse relationship between the two.

The physical coupling of an antibody restricts its movement and the degree to which this influences the reaction kinetics depends upon the nature of the solid-phase surface and on the surface area of coupled antibody in relation to the volume and concentration of the other immunoreactants. As mentioned above the intrinsic antigen:antibody reaction rates are lower for surface reactions than in free solution; the forward rate constant is reduced but the reverse is increased which can result in an overall increase in the equilibrium association constant. However due to the essentially irreversible nature of this binding interaction, the surface antigen:antibody can be considered multivalent thus significantly increasing chances of reassociation; overall reaction rates can become limited by the rate of diffusion of the solution phase component as the surface concentrations become depleted.

The significance of diffusion effects will depend upon the geometry of the solid-phase surface, the intrinsic reaction rate and the surface concentration of antigen:antibody. The geometry is determined by particle size/surface area and the surface concentration by the choice of surface and coupling technique with the intrinsic reaction rate determined by the choice of antibody. Diffusion effects are considered to be limiting only when the particle size of the solid-phase excedes 40 μm, that in general means large balls, paddles, tubes and microtitre plates. Membranes can be used to actively concentrate immunoreactants increasing reaction rates (as described in Chapter 21) due to the large surface areas formed by the vast numbers of individual fibres. Microtitre plates will in general provide more favourable reaction kinetics than tubes as the local concentration of immunoreactants can be higher than in the larger volumes used in tube assays.

Antigen coated solid-phases can also be used; again care is required with larger protein molecules, and even some smaller peptides, as the partial denaturation that will occur on coupling to the solid-phase will alter the expression of epitopes. However this effect has been used to advantage in an enzyme-immunoassay for glycated haemoglobin (Hb A_{1c}), where the binding of the haemoglobin to the surface of a microtitre-plate exposes the glycated epitope (22). Antigen coated matrices have often been used in limited reagent systems and as such are most appropriate to the measurement of small molecules *e.g.* haptens. However the recent development of anti-idiotypic antibodies has now enabled the development of excess-reagent systems for these same small molecules (23).

Washing, NSB and matrix effects

During the development of an immunoassay the optimisation of the reaction and washing buffers is of prime concern in reducing NSB and the influence of the biological matrix (24). The inclusion of buffer salts, chaotropes, proteins *e.g.* BSA and detergents *e.g.* Tween 20 is well known in most immunoassay development.

Most pertinent to this discussion is the effect of the buffer used in the washing step. In Figure 4.1. the effect of repeated washing on the precision profile for a TSH immunoradiometric assay (IRMA) is shown.

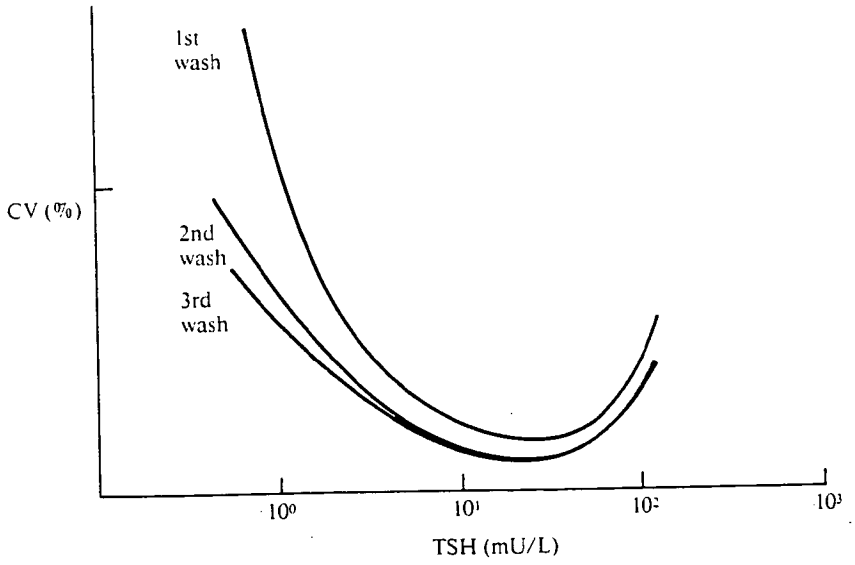

Fig. 4.1. The effect of increasing the number of washing steps on an excess reagent assay precision profile.

This demonstrates the improvement in detection limit and working range that wash steps can introduce, brought about essentially by reducing the NSB. The physical flushing that introducing a wash buffer produces is only one component of this effect, the inclusion of much the same agents, as mentioned above for the reaction buffer, are also important. Washing removes entrapped label, reduces adsorption of label to surfaces and aids removal of the reaction supernatant. When using manual assays there was

the choice as to whether to aspirate or decant the supernatant; despite early doubts concerning the reliability of decanting this is now the prefered technique, not only on the grounds of convenience but also of improved assay precicison.

The manner in which the wash buffer is applied will vary depending upon the solid-phase matrix used. Particles, beads and microtitre plates can be washed actively but in general membranes will be washed by capiliary flow or 'radial partition'. When the flow rate is slow, as in the latter case, the use of detergents and proteins in the wash fluid are even more important in reducing NSB.

Washing and separation steps have been automated in a variety of systems *e.g.* microtitre plates (various commercial plate washers), membranes (Abbott Laboratories ImX®, Opus-PB®), magnetic particles (Serono-Baker™ SRI®, Tosoh, AIA 600®, and the Technicon, Immuno-1®) and coated tubes (Boehringer, ES-600® and the Becton-Dickinson, Affinity®). The automation of these vital parts of a heterogeneous immunoassay have been amongst the most important developments in automating immunoassay (25) (see Chapter 8 for further discussion) .

PARTICULATE SOLID-PHASES

Particulate solid-phases can be roughly divided into two types, magnetic and non-magnetic.

Non-magnetic particulate matrices

These include latex, glass, Sepharose, Sephadex, Sephacryl and nylon particles and beads. The choice between them is based on the relative coupling capacities of the different plastics and the size and density of the particles. The agaroses *e.g.* Sephadex, and celluloses have higher antibody binding capacities than polystyrene, nylon or glass although the absolute capacity per gram of particles will clearly depend upon the surface area available *i.e.* on the size of the individual particles. On the basis of size, smaller polystyrene and glass particles can be more easily made. The choice of appropriate size will, as mentioned previously, depend upon the ease of use and reaction kinetics that are required (see Table 4.2.). Examples of non-magnetic particle systems in widespread use are the single large (0.5

cm) nylon beads used in the kits from the Nichols Institute and in the Olympus automated immunoassay analyser, the Sucrosep® separation system used in the Boots-Celltech range of assays and the microcrystalline celluose system described by Chapman *et al* (14). The Sucrosep sytem used Sepharose beads in combination with a sucrose 'wash' solution which prevented the need for a centifugation step, this procedure was extremely efficient as a separation system (NSB's <0.1%) but cumbersome to handle (26).

There are examples of particles used in combination with other matrices. These include the Abbott ImX which uses primary antibody coated latex particles, to perform the primary antigen:antibody reaction and captures these particles on a glass-fibre membrane for separation and washing (27). Another combination approach is the use of antigen coated particles as an affinity column, which acts both as a separation system and as part of the primary antigen:antibody reaction. Affinity column mediated immunoassay (ACMIA) requires careful control of the elution step and as such is best performed in an automated fashion as exemplified by Freytag *et al* (28). A further example of an automated particle heterogeneous immunoassay was the use of the Centria® centrifugal analyser which was used to automate a centrifugal separation step (29).

Particle based separation systems have been used with both hapten and protein assays and with all detection systems so far described for heterogeneous immunoassays.

Magnetic or magnetisable matrices

Magnetic or magnetisable particles have long been the favourite means for immunoassayists to avoid the use of a centrifugation separation system (30,31). The use of magnetic supports for use in immunoassay dates back to the early 1970's. The advantages of a magnetic particle are a high surface area, rapid analyte capture and properties that lead to efficient separation and washing. The most commonly used magnetic particle has been that made from paramagnetic ferrous oxide (32) and described by Forrest and Landon and used by Serono, Corning and other commercial companies (33-36). The ferrous oxide is incorporated into a cellulose matrix, to which the antibody is coupled, to provide a stable reagent with low NSB. This was extended to develop a magnetisable charcoal reagent by co-trapping charcoal and ferrous oxide in a polyacrylamide gel. This approach

considerably enhanced the use of charcoal as a separation matrix, for hapten assays, in that it removed the centrifugation step but also in that there was less effect on the primary antigen: antibody reaction (37).

The ferrous oxide has proved superior to other magnetic components mainly due to its small particle size (10-20 nm) combined with a good magnetic response; the size is however increased dramatically during entrapment in the cellulose, the final particle size being determined by milling (1-3μm). There have been relatively few advances in magnetic particle technology apart from the introduction of chromium dioxide particles in 1987 (38). Deriving from audio tape technologies, CrO_2 was suggested to offer less residual magnetism than ferrous oxide particles, *i.e.* offering better resuspension kinetics, and low NSB was achievable without incorporation into a polymer matrix and thus an overall smaller particle size of <5 μm without milling (9). The low NSB characterisitics of these particles are such that have recently been used in a non-magnetic ACMIA format in a whole-blood assay for cyclosporin (39).

The first magnetic particle assays used either batch processing in magnetic racks (*e.g.* Corning), or automation in continuous flow systems. More recent automation has included unit dose systems such as the Serono-Baker SRI, and the Tosoh AIA-600 in addition to the Technicon Immuno-1 format multi-dose system that has not yet been commercialised. Magnetic particles have been used in assays as secondary antibody separation systems and coupled to primary antibodies, for use in assays for haptens and proteins using a range of detection systems from isotopes, enzymes and fluorophores to chemiluminescence (Magic-lite® from Ciba-Corning).

SOLID SURFACE MATRICES

The choice of a macro-solid phase is in general determined by a desire to simplify an assay protocol as much as possible. The choice of which plastic support to select is determined by the antibody binding characteristics provided and the desired reaction kinetics. Macro-solid phases come in several formats described below.

Fibres (membranes)

Two types of fibre have been used in immunoassays; firstly cellulose (or

nitro-cellulose) *e.g.* filter paper, and secondly glass-fibre; both are used as part of larger membranous supports. The use of fibrous membranes provides a high surface area with high antibody binding capacity. Direct coupling to cellulose is simple and has been mentioned above; coupling to glass-fibre is also simple and reproducible.

Cellulose membranes have been used in a wide variety of systems employing a variety of washing sytems. The Dade Stratus uses radial partition to wash an antibody coated central portion of a glass-fibre membrane (40) (see also Chapter 22). The Hybritech ICON® uses monoclonal antibodies coupled to a cellulose matrix washed by flow encouraged by an adsorbent layer below the membrane (41) (see also Chapter 21). Monoclonal antibodies have also been coupled to cellulose membranes in the Clearview™ (42), nitro-cellulose in the FIAX®system (43) and cellulose again in the immunochromatography systems (44) (see also Chapter 20).

Nitro-cellulose membranes are commonly used in Western blotting and this approach is extended to the so called Dot-ELISA's. These have been mainly limited to infectious disease assays so far but offer great potential for providing a simple multi-analyte approach by immobilising several antibody 'spots' on a single strip of membrane (45).

Coated tubes

Coated tubes are one of the lowest capacity solid phases used in immunoassay, although one of the earliest to be exploited (10). Along with large single beads and microtitre plates their effective use is critically dependent upon the reproducibility of coupling of the antibody to each tube. Difficulties in achieving this led to very high rejection rates of batches of tubes in order to ensure satisfactory precision in the final assay. Coated tube systems are in commercial use and have been automated by a number of manufacturers especially Boehringer in their ES series (46). The low capacity of the solid phase can produce extremely low NSB's, values of less than 0.01% are achievable although these are not routinely available as coated tube enzyme-labelled systems rarely use 'total' tubes, which are needed to assess NSB.

Analagous to the use of coated tubes is the use of 'dip sticks' which have not been widely used but are best suited to qualitative or semi-quantitative

assays but offer extremely simple protocols (16,47).

Microtitre plates

Microtitre plates are probably the most popular solid phase in use at the present time despite the fact that they also have the lowest antibody binding capacity. Plates are avaiiable in a range of plastics treated in a variety of manners (16,47). The use of polystyrene plates that have been irradiated has been recommended by several authors (16,47), with the capacity of the plates further enhanced by drying the antibody onto the plate. The standard format is the 96 well plate but strips and even individual wells are available. Although microtitre plates have been used with radiolabels (by cutting up the plates) they are most commonly used with non-isotopic systems employing enzyme, chemiluminescent and fluorescent labels. The widespread application of microtitre plates required the development of suitable plate based detection systems and it was delays in the development of these instruments that held back their introduction as long as it did. One great advantage of microtitre plates that was immediately apparent to the immunoassaysist was that less labelling of the tube/plate was required, reducing a very tedious and labour intensive activity.

Difficulties with the plate format have included drift across the plate due to pipetting delays and temperature gradients across the plates due to their thermal insulating qualities. Batch to batch difficulties in preparation are also a problem and careful attention to quality control is required by commercial plate manufacturers. Due to the low capacity of the solid phase reaction kinetics have been improved by incubating at elevated temperatures (37°C) and using continuous vibration. Combinations of these approaches have resulted in extremely sensitive assays for proteins in only a few hours *e.g.* the enzyme-amplified TSH assay using a kinetic plate reader (48). The microtire plate format does not lend itself to complete automation but there have been significant advances in work simplification that have resulted in partial automation.

CONCLUSIONS

A wide variety of solid phase immunological separation systems have been developed and highly sensitive assays using a variety of detection systems have been acheived. Due to the use of washing techniques it is probably fair to say that it is the detection systems that limit the sensitivity of the assays

(both excess and limited reagent) and not the capacity of the solid phase. The solid phase can however dictate the flexibility in the delivery system.

FUTURE PROSPECTS

There are further advances in heterogeneous immunoassay that now extends to dry film technologies (49). The development of 'ambient analyte' assays (see Chapter 5) offers a challenge to the classical separation between heterogeneous and homogeneous immunoassays; whilst requiring solid phase technology it is inappropriate to consider its use as that of a separation system (see Chapter 5). A similar position applies with the surface effect immunoassay techniques such as surface plasmon resonance and ellipsometry where the reaction is monitored 'through' the solid-phase matrix to which the immunoreactant is coupled and thus the 'solid-phase' is an integral part of the measuring system (see Chapter 18). The future of immunoassay may thus not include separation as we know it.

REFERENCES

1. Ratcliffe, J.G. (1974) Separation techniques in saturation analysis. *Brit. Med. Bull.* **30**, 32-7.
2. Hunter, W.M. and Corrie, J.E.T. (eds.) (1983) *Immunoassays for Clinical Chemistry*. 2nd Edn. pp. 701, Churchill-Livingstone, Edinburgh.
3. Gosling, J.P. (1990) A decade of development in immunoassay methodology. *Clin. Chem.* **36**, 1408-27.
4. Wide, L. and Porath, J. (1966) Radioimmunoassays of proteins with the use of Sephadex coupled antibodies. *Biochim. Biophys. Acta.* **130**, 257-62.
5. Miles, L.E.M. and Hales, C.N. (1968) Labelled antibodies and immunological assay systems. *Nature.* **219**, 186-9.
6. Utiger, R.D., Parker, M.L., Daughaday, W.H. (1962) Studies on human growth hormone. I A radioimmunoassay for human growth hormone. *J. Clin. Invest.* **41**, 254-61.
7. Edwards, R. (1983) The development and use of a PEG assisted second-antibody as a separation technique in RIA. In: *Immunoassays for Clinical Chemistry*. (eds. Hunter, W.M. and Corrie, J.E.T.) 2nd Edn, p 139-46, Churchill-Livingstone, Edinburgh.

8. Oliver, J.R., Hakendorf, P., Zeegers, P., Ross, W. (1982) A proposed simple method for detection and measurement of antibodies to insulin in serum by use of staphylococcus aureus containing protein A. *Clin. Chem.* **28**, 121-3.
9. Newman, D.J., Medcalf, E.A., Gorman, E.G., Price, C.P. (1989) A novel solid phase enzyme-immunoassay for beta-2-microglobulin. Biologie Prospective. Comptes rendus de 7e Colloque de Ponte-a-Mousson. (eds. Galteau, M-M., Siest, G., Henny, J.) p 119-22, John Libbey, Eurotext.
10. Catt, K. and Tregear, C.W. (1967) Solid-phase radioimmunoassay in antibody coated tubes. *Science.* **158**, 1570-2.
11. Donini, S. and Donini, P. (1969) Radioimmunoassay employing polymerised antisera. *Acta. Endocrinol.* **Suppl. 142**, 25-8.
12. Bolton, A.E.and Hunter, W.M. (1973) The use of antisera covalently coupled to agarose, cellulose and Sephadex in radioimmunoassays for proteins and haptens. *Biochim. Biophys. Acta.* **329**, 318-30.
13. Axon, R., Porath, J., Ernback, S. (1967) Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen bromide. *Nature.* **214**, 1302-4.
14. Chapman, R.S., Sutherland, R.M., Ratcliffe, J.G. (1983) Application of 1,1'-carbonyldiimidazole as a rapid practical method for the production of solid-phase immunoassay reagents. In: *Immunoassays for Clinical Chemistry.* (eds. Hunter, W.M. and Corrie, J.E.T.) 2nd Edn, p 178-90, Churchill-Livingstone, Edinburgh.
15. Wood, W.G., and Gadow, A. (1983) Immobilisation of antibodies and antigens on macro solid phases: a comparison between adsorptive and covalent binding. Part 1 of a critical study of macro solid phases for use in immunoassay systems. *J. Clin. Chem. Clin. Biochem.* **21**, 789-97.
16. Kemeny, D.M. and Challacombe, S.J. (1988) Microtitre plates and other solid-phase supports. In: *ELISA and other Solid Phase Immunoassays: Theoretical and Practical Aspects.* (eds. Kemeny, D.J. and Challacombe, S.J.) p 31-56, John Wiley and Sons, Chichester.
17. Thakkar, H., Davey, C.L., Medcalf, E.A., et al. (1991) Stabilisation of turbidimetric immunoassay by covalent coupling of antibody to latex particles. *Clin. Chem.* , in press.
18. Voller, A., Bidwell, D.E., Bartlett, A. (1979) *The Enzyme-linked Immunosorbent Assay (ELISA).* Dynatech Europe, UK.
19. Engvall, E. and Perlmann, P. (1971) Enzyme-linked immunosorbent

assay (ELISA): quantitative assay of IgG. *Immunochemistry*. **8**, 871.
20. Nygren, H. and Stenberg, A.M. (1989) Immunochemistry at interfaces. *Immunology*. **66**, 321-7.
21. Stenberg, M. and Nygren, H. (1988) Kinetics of antigenantibody reactions at solid-liquid interfaces. *J. Immunol. Meth.* **113**, 3-15.
22. Engbaek, F., Christensen, S.E., Jespersen, B. (1989) Enzyme immunoassay of haemoglobin A_{1c}: analytical characteristics and clinical performance for patients with diabetes mellitus, with and without uremia. *Clin. Chem.* **35**, 93-7.
23. Barnard, G. and Kohen, F. (1990). Idiometric assay: non competitive immunoassay for small molecules typified by the measurement of estradiol in serum. *Clin. Chem.* **36**, 1945-50.
24. Wood, W. (1991) Matrix effects in immunoassays. *Scand. J. Clin. Lab. Invest.* **51**, Suppl. 205, 105-12.
25. Ehrhardt, V., Neumeier, D., Meyer, H.D. (1988) Mechanization of heterogeneous immunoassays. *J. Clin. Immunoassay*. **11**, 74-80.
26. Wright, J.F. and Hunter, W.M. (1983) The sucrose layering separation: A non-centrifugation system. In: *Immunoassays for Clinical Chemistry*. (eds. Hunter, W.M. and Corrie, J.E.T.) 2nd Edn, p 170-7, Churchill-Livingstone, Edinburgh.
27. Fiore, M., Mitchell, J., Doan, T., et al. (1988) The Abbott ImX™ automated benchtop immunochemistry analyzer system. *Clin. Chem.* **34**, 1726-32.
28. Freytag, J.W., Dickinson, J.C., Tseng, S.Y. (1984) A high sensitivity affinty-column-mediated immunometric assay as exemplified by digoxin. *Clin. Chem.* **30**, 417-20.
29. Mériadec, B., Jolu, J-P., Henry, R. (1979) A new and universal separation system technique for the 'Centria' automated radioimmunoassay system. *Clin. Chem.* **25**, 1596-9.
30. Forrest, G.C. and Rattle, S.J. Magnetic particle radioimmunoassay. In: *Immunoassays for Clinical Chemistry*. (eds. Hunter, W.M. and Corrie, J.E.T.) 2nd Edn, p 147-62, Churchill-Livingstone, Edinburgh.
31. Hersh, L.S. and Yaverbaum, S. (1975) Magnetic solid phase radioimmunoassay. *Clin. Chim. Acta*. **63**, 69-72.
32. Robinson, P.J., Dunhill, P., Lilly, M.D. (1973) The properties of magnetic supports in relation to immobilized enzyme reagents. *Biotech. Bioeng*. **15**, 603-6.
33. Forrest, G.C. (1977) Development and application of a fully automated continuous flow radioimmunoassay system. *Ann. Clin.*

Biochem. **14**, 1-11.

34. Nargessi, R.D., Landon, J., Pourfarzaneh, M., Smith, D.S. (1978) Solid phase fluoroimmunoassay of human albumin in biological fluids. *Clin. Chim. Acta.* **89**, 455-60
35. Harmer, I.J. and Samuel, D. (1989) The FITC-anti-FITC system is a sensitive alternative to biotin-streptavidin in ELISA. *J. Immunol. Method.* **122**, 115-21.
36. Milne, C.N., Pritchard, G.J., Allen, G.J., et al. (1988) Automation of enzyme immunoassays for hormones. *J. Endocr.* **119**, Suppl. Abstract, 89.
37. Al-Dujaili, E.A.S., Forrest, G.C., Edwards, C.R.W., Landon, J. (1979) Evaluation and application of magnetizable charcoal for separation in radioimmunoassays. *Clin. Chem.* **25**, 1402-5.
38. Birkmeyer, R.C., Diaco, R., Hutson, D.K., et al. (1987) Application of novel chromium dioxide magnetic particles to immunoassay development. *Clin. Chem.* **33**, 1543-7.
39. Hansen, J.B., Lay, H.P., Janes, C.J., et al. (1990) A rapid and specific assay for the du Pont aca discrete clinical analyser, performed directly on whole blood. *Transpl. Proc.* **22**, 1189-92.
40. Giegel, J.L., Brotherton, M.M., Cronin, P., et al. (1982) Radial partition immunoassay. *Clin. Chem.* **28**, 1894-8.
41. Valkirs, G.E. and Barton, R. (1985) ImmunoConcentration™ - a new format for solid-phase immunoassays. *Clin. Chem.* **31**, 1427-31
42. Unipath Clear Blue pregnancy test, Unipath-Oxoid, Bedford, UK.
43. Wang, R., Merrill, B., Maggio, E.T. (1980) A simplified solid phase immunofluorescence assay for measurement of serum immunoglobulins. *Clin. Chim.Acta.* **102**, 169-77.
44. Zuk, R. F., Ginsberg, V. K., Houts, T., et.al. (1985) Enzyme immunochromatography a quantitative immunoassay requiring no instrumentation. *Clin. Chem.* **31**, 1144-50.
45. Pappas, M.G. (1988) Dot enzyme-linked immunosorbent assays. In: *Complementary Immunoassays.* (ed Collins, W.P.) p 113-34, John Wiley and Sons., Chichester.
46. Chan, D.W., Waldron, C., Bill, M.J., Drew, H. (1987) The performance of a totally automated enzyme immunoassay system (ES 600). *Clin. Chem.* **33**, 947. [Abstract].
47. Rasmussen, S.E. (1988) Solid phases and chemistries. In: *Complementary Immunoassays.* (ed Collins, W.P.) p 43-55, John Wiley and Sons., Chichester.
48. Clark, P.M.S., and Price, C.P. (1986) Enzyme amplified

immunoassays: a new ultrasensitive assay of thyrotropin evaluated. *Clin. Chem.* **32**, 88-92.

49. Hiratsuka, N., Mihara, Y. and Miyazako, T. (1982) Method for immunological assay using multilayer analysis sheet. *U. S. Patent No.* **4,337,065.**